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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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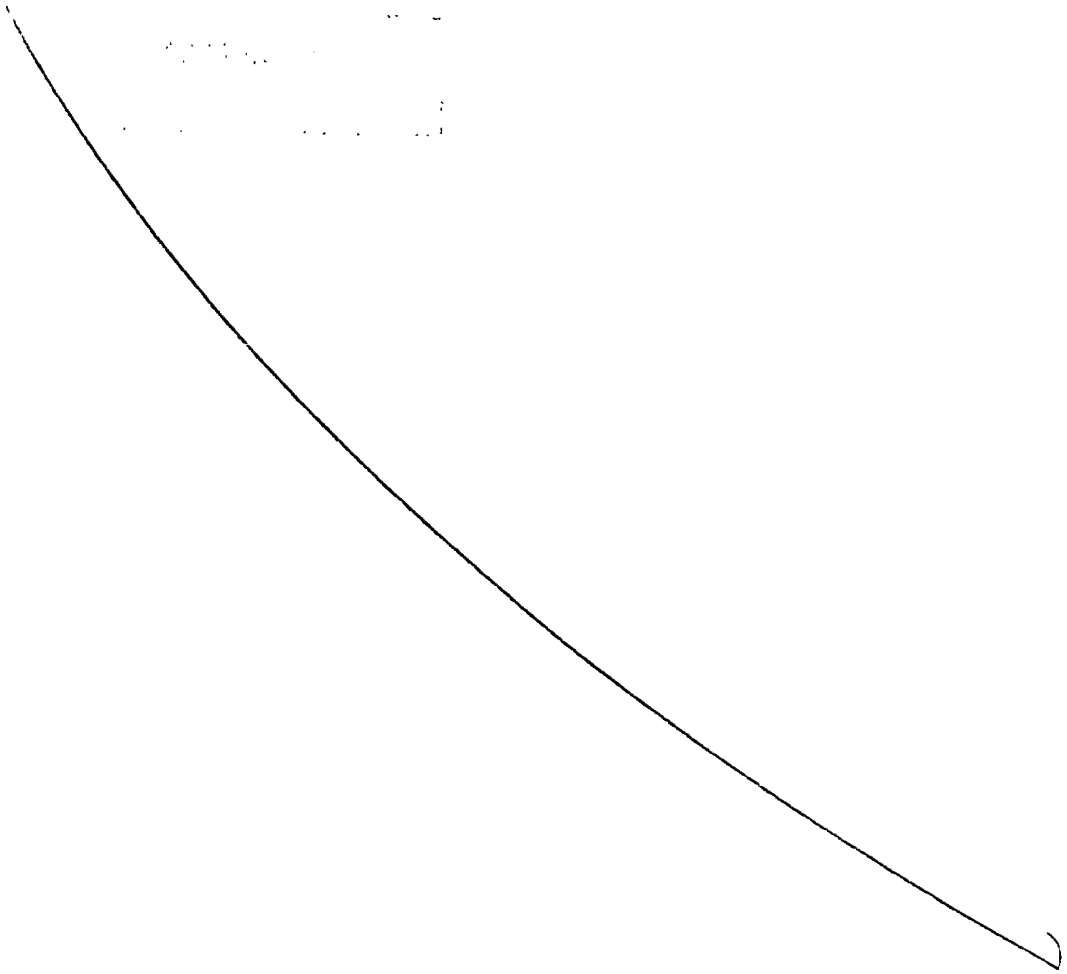

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Sheet 2 of the certificate
Page 2 de l'attestation

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Selecting animals for parentally imprinted traits

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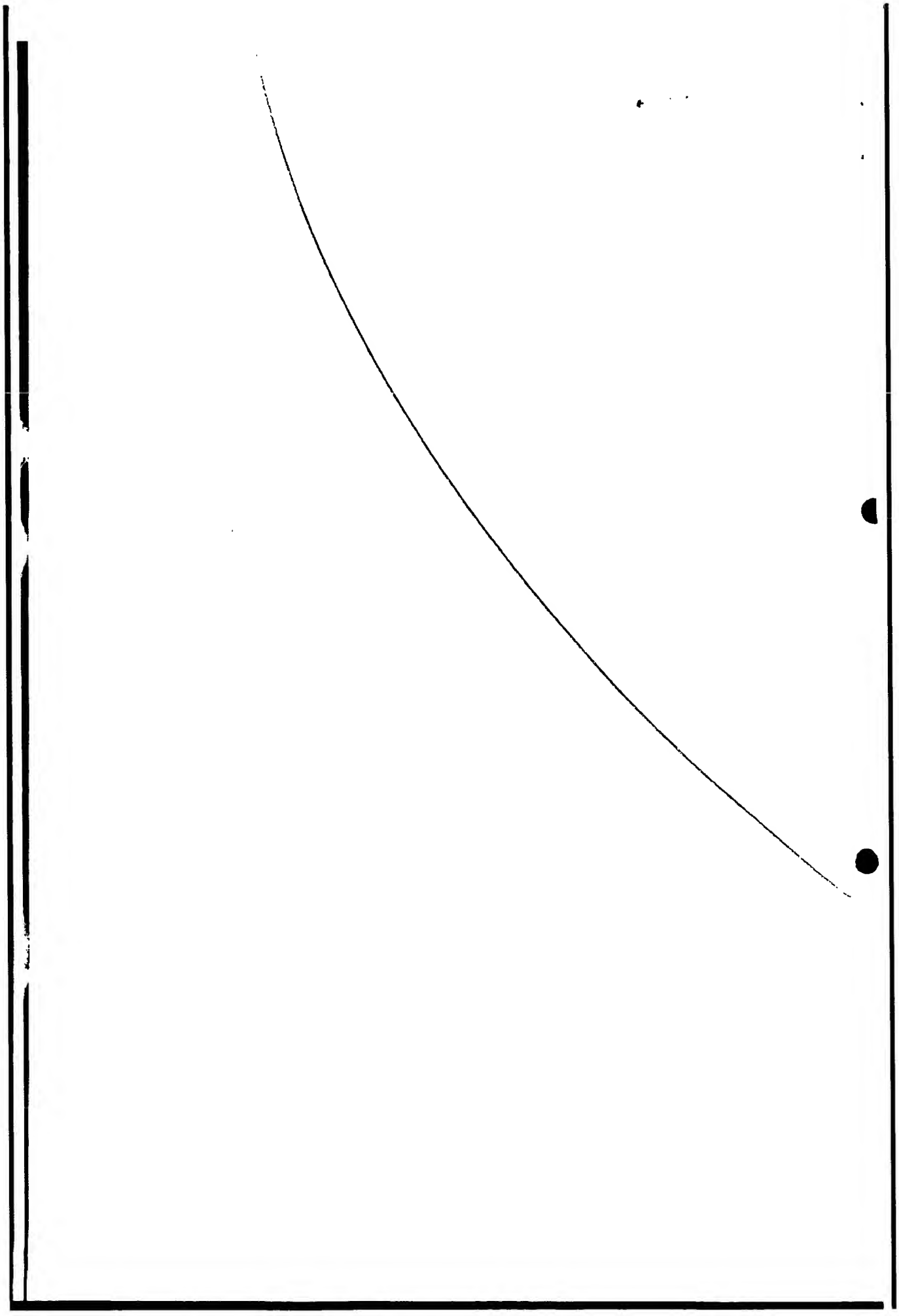

Title: Selecting animals for parentally imprinted traits.

The invention relates to methods to select breeding animals or animals destined for slaughter for having desired genotypic or potential phenotypic properties, in particular related to muscle mass and/or fat deposition. Breeding schemes for domestic animals have so far focused on farm performance traits and carcass quality. This has resulted in substantial improvements in traits like reproductive success, milk production, lean/fat ratio, prolificacy, growth rate and feed efficiency. Relatively simple performance test data have been the basis for these improvements, and selected traits were assumed to be influenced by a large number of genes, each of small effect (the infinitesimal gene model). There are now some important changes occurring in this area. First, the breeding goal of some breeding organisations has begun to include meat quality attributes in addition to the "traditional" production traits. Secondly, evidence is accumulating that current and new breeding goal traits may involve relatively large effects (known as major genes), as opposed to the infinitesimal model that has been relied on so far.

Modern DNA-technologies provide the opportunity to exploit these major genes, and this approach is a very promising route for the improvement of meat quality, especially since direct meat quality assessment is not viable for potential breeding animals.

The evidence for several of the major genes originally obtained using segregation analysis, i.e. without any DNA marker information. Afterwards molecular studies were performed to detect the location of these genes on the genetic map. In practice, and except for alleles of very large effect, DNA studies are required to dissect the genetic nature of most traits of economic importance. DNA markers can be used to localise genes or alleles responsible for qualitative traits like coat colour, and they can also be used to detect genes or

alleles with substantial effects on quantitative traits like growth rate, IMF etc. In this case the approach is referred to as QTL (quantitative trait locus) mapping, wherein a QTL comprises at least a part of the nucleic acid genome of an animal where genetic information capable of influencing said quantitative trait (in said animal or in its offspring) is located. Information at DNA level can not only help to fix a specific major gene in a population, but also assist in the selection of a quantitative trait which is already selected for. Molecular information in addition to phenotypic data can increase the accuracy of selection and therefore the selection response.

Improving meat quality is not just about changing levels of traits like tenderness or marbling, but it is also about increasing uniformity. The existence of major genes provides excellent opportunities for improving meat quality because it allows large steps to be made in the desired direction. Secondly, it will help to reduce variation, since we can fix relevant genes in our products. Another aspect is that selecting for major genes allows differentiation for specific markets. Studies are underway in several species, particularly, pigs, sheep, deer and beef cattle.

In particular, intense selection for meat production has resulted in animals with extreme muscularity and leanness in several livestock species. In recent years it has become feasible to map and clone several of the genes causing these phenotypes, paving the way towards more efficient marker assisted selection, targeted drug development (performance enhancing products) and transgenesis. Mutations in the ryanodine receptor (Fuji et al, 1991; MacLennan and Phillips, 1993) and myostatin (Grobet et al, 1997; Kambadur et al, 1997; McPherron and Lee, 1997) have been shown to cause muscular hypertrophies in pigs and cattle respectively, while genes with major effects on muscularity and/or fat deposition have for instance been mapped to pig

chromosome 4 (Andersson et al, 1994) and sheep chromosome 18 (Cocket et al, 1996).

However, although there have been successes in identifying QTLs, the information is currently of limited use within commercial breeding programmes. Many workers in this field conclude that it is necessary to identify the particular genes underlying the QTL. This is a substantial task, as the QTL region is usually relatively large and may contain many genes. Identification of the relevant genes from the many that may be involved thus remains a significant hurdle in farm animals.

The invention provides a method for selecting a domestic animal for having desired genotypic or potential phenotypic properties comprising testing said animal for the presence of a parentally imprinted qualitative or quantitative trait locus (QTL). Herein, a domestic animal is defined as an animal being selected or having been derived from an animal having been selected for having desired genotypic or potential phenotypic properties.

Domestic animals provide a rich resource of genetic and phenotypic variation, traditionally domestication involves selecting an animal or its offspring for having desired genotypic or potential phenotypic properties. This selection process has in the past century been facilitated by growing understanding and utilisation of the laws of Mendelian inheritance. One of the major problems in breeding programs of domestic animals is the negative genetic correlation between reproductive capacity and production traits. This is for example the case in cattle (a high milk production generally results in slim cows and bulls) poultry, broiler lines have a low level of egg production and layers have generally very low muscle growth), pigs (very prolific sows are in general fat and have comparatively less meat) or sheep (high prolific breeds have low carcass quality and vice versa). The invention now provides that knowledge of the

parental imprinting character of various traits allows to select for example sire lines homozygous for a paternally imprinted QTL for example linked with muscle production or growth; the selection for such traits can thus be less stringent in dam lines in favour of the reproductive quality. The phenomenon of genetic or paternal imprinting has never been utilised in selecting domestic animals, it was never considered feasible to employ this elusive genetic characteristic in practical breeding programmes. The invention provides a breeding programme, wherein knowledge of the parental imprinting character of a desired trait, as demonstrated herein, results in a breeding programme, for example in a BLUP programme, with a modified animal model. This increases the accuracy of the breeding value estimation and speeds up selection compared to conventional breeding programmes. Until now, the effect of a parentally imprinted trait in the estimation of a conventional BLUP programme was neglected; using and understanding the parental character of the desired trait, as provided by the invention, allows selecting on parental imprinting, even without DNA testing.

In a preferred embodiment, the invention provides a method for selecting a domestic animal for having desired genotypic or potential phenotypic properties comprising testing a nucleic acid sample from said animal for the presence of a parentally imprinted quantitative trait locus (QTL). A nucleic acid sample can in general be obtained from various parts of the animal's body by methods known in the art. Traditional samples for the purpose of nucleic acid testing are blood samples or skin or mucosal surface samples, but samples from other tissues can be used as well, in particular sperm samples, oocyte or embryo samples can be used. In such a sample, the presence and/or sequence of a specific nucleic acid, be it DNA or RNA, can be determined with methods known in the art, such as hybridisation or nucleic acid amplification or sequencing techniques known in the art.

The invention provides testing such a sample for the presence of nucleic acid wherein a QTL or allele associated therewith is associated with the phenomenon of parental imprinting, for example where it is determined whether a paternal or maternal allele of said QTL is capable of being predominantly expressed in said animal.

The purpose of breeding programs in livestock is to enhance the performances of animals by improving their genetic composition. In essence this improvement accrues by increasing the frequency of the most favourable alleles for the genes influencing the performance characteristics of interest. These genes are referred to as QTL. Until the beginning of the nineties, genetic improvement was achieved via the use of biometrical methods, but without molecular knowledge of the underlying QTL.

Since the beginning of the nineties and due to recent developments in genomics, it is conceivable to identify the QTL underlying a trait of interest. The invention now provides identifying and using parentally imprinted QTLs.

The invention provides the initial localisation of a parentally imprinted QTL on the genome by linkage analysis with genetic markers, and the actual identification of the parentally imprinted gene(s) and causal mutations therein. Molecular knowledge of such a parentally imprinted QTL allows for more efficient breeding designs herewith provided. Applications of molecular knowledge of parentally imprinted QTLs in breeding programs include: marker assisted segregation analysis to identify the segregation of functionally distinct parentally imprinted QTL alleles in the populations of interest, marker assisted selection (MAS) performed within lines to enhance genetic response by increasing selection accuracy, selection intensity or by reducing the generation interval using the understanding of the phenomonon of parental imprinting, marker assisted introgression (MAI) to efficiently transfer favourable parentally imprinted QTL alleles from a donor to a

recipient population, genetic engineering of the identified parentally QTL and genetic modification of the breeding stock using transgenic technology, development of performance enhancing products using targeted drug development exploiting molecular knowledge of said QTL.

The inventors undertook two independent experiments to determine the practical use of parental imprinting of a QTL.

In a first experiment, performed in a previously described Piétrain x Large White intercross, the likelihood of the data were computed under a model of paternal (paternal allele only expressed) and maternal imprinting (maternal allele only expressed) and compared with the likelihood of the data under a model of a conventional "Mendelian" QTL. The results strikingly demonstrated that the QTL was indeed paternally expressed, the QTL allele (Piétrain or Large White) inherited from the F1 sow having no effect whatsoever on the carcass quality and quantity of the F_2 offspring. It was seen that very significant lodscores were obtained when testing for the presence of a paternally expressed QTL, while there was no evidence at all for the segregation of a QTL when studying the chromosomes transmitted by the sows. The same tendency was observed for all traits showing that the same imprinted gene is responsible for the effects observed on the different traits. Table 1 reports the maximum likelihood (ML) phenotypic means for the F_2 offspring sorted by inherited paternal QTL allele.

In a second experiment performed in the Wild Boar X Large White intercross, QTL analyses of body composition, fatness, meat quality, and growth traits was carried out with the chromosome 2 map using a statistical model testing for the presence of an imprinting effect. Clear evidence for a paternally expressed QTL located at the very distal tip of 2p was obtained (Fig. 2; Table1). The clear paternal expression of a QTL is illustrated by the least squares means which fall into two classes following the population origin of the paternally inherited allele

(Table 1). For a given paternally imprinted QTL, implementation of marker assisted segregation analysis, selection (MAS) and introgression (MAI), can be performed using genetic markers that are linked to the QTL, genetic markers that are in linkage disequilibrium with the QTL, or using the actual causal mutations within the QTL.

Understanding the parent-of-origin effect characterising a QTL allows for its optimal use in breeding programs. Indeed, marker assisted segregation analysis under a model of parental imprinting will yield better estimates of QTL allele effects. Moreover it allows for the application of specific breeding schemes to optimally exploit a QTL. In one embodiment of the invention, the most favourable QTL alleles would be fixed in breeding animal lines and for example used to generate commercial, crossbred males by marker assisted selection (MAS, within lines) and marker assisted introgression (MAI, between lines). In another embodiment, the worst QTL alleles would be fixed in the animal lines used to generate commercial crossbred females by MAS (within lines) and MAI (between lines).

In a preferred embodiment of the invention, said animal is a pig. Note for example that the invention provides the insight that today half of the offspring from commercially popular Piétrain$_x$ Large White crossbred boars inherit an unfavourable Large White muscle mass QTL as provided by the invention causing considerable loss, and the invention now for example provides the possibility to select the better half of the population in that respect. However, it is also possible to select commercial sow lines enriched with the in the boars unfavourable alleles, allowing to equip the sows with other alleles more desirable for for example reproductive purposes.

In a preferred embodiment of a method provided by the invention, said QTL is located at a position corresponding to a QTL located at chromosome 2 in the pig. For example, it is known form comparative mapping data between pig and human, including bidirectional

chromosome painting, that SSC2p is homologous to HSA11pter-q13[11,12]. HSA11pter-q13 is known to harbour a cluster of imprinted genes: IGF2, INS2, H19, MAH2, $P57^{KIP2}$, K_vLQTL1, Tapa1,/CD81, Orctl2, Impt1 and Ip1. The cluster of imprinted genes located in HSA11pter-q13 is characterised by 8 maternally expressed genes H19, MASH2, $P57^{KIP2}$, K_vLQTL1, TAPA1/CD81, ORCTL2, IMPT1 and IP1, and two paternally expressed genes: IGF2 and INS.

Other domestic animals, such as cattle, sheep, poultry and fish, have similar homologous regions in their genome harbouring such a cluster of imprinted genes or QTLs, the invention herewith provides use of these orthologous regions of other domestic animals in applying the phenomenon of parental imprinting in breeding programmes. In pigs, said cluster is mapped at around position 2p1.7 of chromosome 2, however, a method as provided by the invention employing (fragments of) said maternally or paternally expressed orthologous or homologous genes or QTLs are advantageously used in other animals as well for breeding and selecting purposes. For example, a method is provided wherein said QTL is related to the potential muscle mass and/or fat deposition of said animal or wherein said QTL comprises at least a part of an insulin-like growth factor-2 (IGF2) allele.

In a preferred embodiment, the invention provides a method for selecting a pig for having desired genotypic or potential phenotypic properties comprising testing a sample from said pig for the presence of a quantitative trait locus (QTL) located at a Sus scrofa chromosome 2 mapping at position 2p1.7. In particular, the invention relates to the use of genetic markers for the telomeric end of pig chromosome 2p in marker selection (MAS) of a parentally imprinted Quantitative Trait Locus (QTL) affecting carcass yield and quality in pigs. Furthermore, the invention relates to the use of genetic markers associated with the IGF2 locus in MAS in pigs.
In a preferred embodiment, the invention provides a QTL located at the distal tip of *Sus scrofa* chromosomes 2

with effects on varies measurements of carcass quality and quantity, particularly muscle mass and fat deposition.

In a first experiment, a QTL mapping analysis was performed in a Wild Boar X Large White intercross counting 200 F_2 individuals. The F_2 animals were sacrificed at a live eight of at least 80 kg or at a maximum age of 190 days. Phenotypic data on birth weight, growth, fat deposition, body composition, weight of internal organs, and meat quality were collected; a detailed description of the phenotypic traits are provided by Andersson *et al*[1] and Andersson-Eklund *et al*[4].

A QTL on chromosome 2p with moderate effect on muscle mass was previously reported by the inventors. The marker map of chromosome 2p was improved as part of this invention by adding microsatellite markers in order to cover the entire chromosome arm. The following microsatellite markers were used: *Swc9*, *Sw2443*, *Sw2623*, and *Swr2516*, all from the distal end of 2p[7]. QTL analyses of body composition, fatness, meat quality, and growth traits were carried out with the new chromosome 2 map. Clear evidence for a QTL located at the very distal tip of 2p was obtained (Fig. 1; Table 1). The QTL had very large effects on lean meat content in ham and explained an astonishing 30% of the residual phenotypic variance in the F_2 population. Large effects on the area of the longissumus dorsi muscle, on the weight of the heart, and on back-fat thickness (subcutaneous fat) were also noted. A moderate effect on one meat quality trait, reflectance value, was indicated. The QTL had no significant effect on abdominal fat, birth weight, growth, weight of liver, kidney, or spleen (data not shown). The Large White allele at this QTL was associated with larger muscle mass and reduced back-fat thickness consistent with the difference between this breed and the Wild Boar population.

In a second experiment, QTL mapping was performed in a Piétrain X Large White intercross comprising 1125 F_2

offspring. The Large White and Piétrain parental breeds differ for a number of economically important phenotypes. Piétrains are famous for their exceptional muscularity and leanness [10](Figure 2, while Large Whites show superior growth performance. Twenty-one distinct phenotypes measuring growth performance (5), muscularity (6), fat deposition (6), and meat quality (4), were recorded on all F_2 offspring. In order to map QTL underlying the genetic differences between these breeds, the inventors undertook a whole genome scan using microsatellite markers on an initial sample of 677 F_2 individuals. The following microsatellite marker map was used to analyse chromosome 2;:SW2443, SWC9 and SW2623, SWR2516-(0,20)-SWR783-(0,29)-SW240-(0,20)-SW776-(0,08)-S0010-(0,04)-SW1695-(0,36)-SWR308. Analysis of pig chromosome 2 using a Maximum Likelihood multipoint algorithm, revealed highly significant lodscores (up to 20) for three of the six phenotypes measuring muscularity (% lean cuts, % ham, % loin) and three of the six phenotypes measuring fat deposition (back-fat thickness (BFT), % backfat, % fat cuts) at the distal end of the short arm of chromosome 2 (Figure 1). Positive lodscores were obtained in the corresponding chromosome region for the remaining six muscularity and fatness phenotypes, however, not reaching the experiment-wise significance threshold)(α=5%. There was no evidence for an effect of the corresponding QTL on growth performance (including birth weight) or recorded meat quality measurements (data not shown). To confirm this finding, the remaining sample of 355 F_2 offspring was genotyped for the four most distal 2p markers and QTL analysis performed for the traits yielding the highest lodscores in the first analysis. Lodscores ranged from 2.1 to 7.7, clearly confirming the presence of a major QTL in this region. Table 2 reports the corresponding ML estimates for the three genotypic means as well as the residual variance. Evidence based on marker assisted segregation analysis points towards residual segregation at this locus within the Piétrain population.

These experiments therefore clearly indicated the existence of a QTL with major effect on carcass quality and quantity on the telomeric end of pig chromosome arm 2p;

the likely existence of an allelic series at this QTL with at least three alleles: Wild-Boar < Large White < Piétrain, and possibly more given the observed segregation within the Piétrain breed.

The effects of the identified QTL on muscle mass and fat deposition are truly major, being of the same magnitude of those reported for the *CRC* locus though apparently without the associated deleterious effects on meat quality. We estimate that both loci jointly explain close to 50% of the Piétrain versus Large White breed difference for muscularity and leanness. The QTL had very large effects on lean meat content in ham and explained an astonishing 30% of the residual phenotypic variance in the F_2 population. Large effects on the area of the longissumus dorsi muscle, on the weight of the heart, and on back-fat thickness (subcutaneous fat) were also noted. A moderate effect on one meat quality trait, reflectance value, was indicated. The QTL had no significant effect on abdominal fat, birth weight, growth, weight of liver, kidney, or spleen (data not shown). The Large White allele at this QTL, when compared to the Wild Boar allele, was associated with larger muscle mass and reduced back-fat thickness consistent with the difference between this breed and the Wild Boar population. The strong imprinting effect observed for all affected traits shows that a single causative locus is involved. The pleiotropic effects on skeletal muscle mass and the size of the heart appear adaptive from a physiological point of view as a larger muscle mass requires a larger cardiac output.

In a further embodiment, the invention provides a method for selecting a pig for having desired genotypic or potential phenotypic properties comprising testing a

sample from said pig for the presence of a quantitative trait locus (QTL) located at a Sus scrofa chromosome 2 mapping at position 2p1.7., wherein said QTL comprises at least a part of a Sus scrofa insulin-like growth factor-2 (IGF2) allele. The important role of *IGF2* for prenatal development is well-documented from knock-out mice as well as from its causative role in the human Beckwith-Wiedemann syndrome. This invention demonstrates an important role for the *IGF2*-region also for postnatal development.

To show the role of Igf2 the inventors performed the following three experiments:

A genomic IGF2 clone was isolated by screening a porcine BAC library. FISH analysis with this BAC clone gave a strong consistent signal on the terminal part of chromosome 2p.

A polymorphic microsatellite is located in the 3'UTR of IGF2 in mice (GenBank U71085), humans (GenBank S62623), and horse (GenBank AF020598). The possible presence of a corresponding porcine microsatellite was investigated by direct sequencing of the IFG2 3'UTR using the BAC clone. A complex microsatellite was identified about 800bp downstream of the stop codon; a sequence comparison revealed that this microsatellite was identical to a previously described anonymous microsatellite, *Swc9*[6]. This marker was used in the initial QTL mapping experiments and its location on the genetic map correspond with the most likely position of the QTL both in the Piétrain X Large White and in the Large White x Wild Boar pedigree.

Analysis of skeletal muscle and liver cDNA from 10-week old foetuses heterozygous for a nt241 (G-A) transversion in the second exon of the porcine IGFII gene and SWC9, shows that the IGFII gene is imprinted in these tissues in the pig as well and only expressed from the paternal allele.

Based on a published porcine adult liver cDNA sequence[16], the inventors designed primer pairs allowing

to amplify the entire *IgfII* coding sequence with 222 bp of leader and 280 bp of trailor sequence from adult skeletal muscle cDNA. Piétrain and Large White RT-PCR products were sequenced indication that the coding sequences are identical in both breeds and with the published sequence. However, a G→A transition was found in the leader sequence corresponding to exon 2 in man. Following conventional nomenclature, this polymorphism will be referred to as *nt241*(G-A). We developed a screening test for this single nucleotide polymorphism 9(SNP) based on the ligation amplification reaction (LAR), allowing us to genotype our pedigree material. Based on these data, *IgfII* was shown to colocalize with the SWC9 microsatellite marker (θ=0%), therefore virtually coinciding with the most likely position of the QTL, and well within the 95% support interval for the QTL. Subsequent sequence analysis demonstrated that the microsatellite marker SWC9 is actually located within the 3'UTR of the *IgfII* gene.

As previously mentioned, the knowledge of this QTL provides a method for the selection of animals such as pigs with improved carcass merit. Different embodiments of the invention are envisaged, including: marker assisted segregation analysis to identify the segregation of functionally distinct QTL alleles in the populations of interest; marker assisted selection (MAS) performed within lines to enhance genetic response by increasing selection accuracy, selection intensity or by reducing the generation interval; marker assisted introgression (MAI) to efficiently transfer favourable QTL alleles from a donor to a recipient population, thereby enhancing genetic response in the recipient population. Implementation of embodiments marker assisted segregation analysis, selection (MAS) and introgression (MAI), can be performed using genetic markers that are linked to the QTL; genetic markers that are in linkage disequilibrium with the QTL, the actual causal mutations within the QTL.

In a further embodiment, the invention provides a method for selecting a pig for having desired genotypic or potential phenotypic properties comprising testing a sample from said pig for the presence of a quantitative trait locus (QTL) located at a Sus scrofa chromosome 2 mapping at position 2p1.7., wherein said QTL is paternally expressed, i.e. is expressed from the paternal allele. In man and mouse, *Igf2* is known to be imprinted and to be expressed exclusively from the paternal allele in several tissues' Analysis of skeletal muscle cDNA from pigs heterozygous for the SNP and/or SWC9, shows that the same imprinting holds in the pig as well. Understanding the parent-of-origin effect characterising the QTL as provided by the invention now allows for its optimal use in breeding programs. Indeed, today half of the offspring from commercially popular Piétrain x Large White crossbred boars inherit the unfavourable Large White allele causing considerable loss. Using a method as provide by the invention avoids this problem.

The invention furthermore provides an isolated and/or recombinant nucleic acid or functional fragment derived thereof comprising a parentally imprinted quantitative trait locus (QTL) or fragment thereof capable of being predominantly expressed by one parental allele. Having such a nucleic acid as provided by the invention available allows constructing transgenic animals wherein favourable genes are capable of being exclusively or predominantly expressed by one parental allele, thereby equipping the offspring of said animal homozygous for a desired trait with desired properties related to that parental allele that is expressed.

In a preferred embodiment, the invention provides an isolated and/or recombinant nucleic acid or fragment derived thereof comprising a synthetic parentally imprinted quantitative trait locus (QTL) or functional fragment thereof derived from at least one chromosome. Synthetic herein describes a parentally expressed QTL wherein various elements are combined that originate from

distinct locations from the genome of one or more animals. The invention provides recombinant nucleic acid wherein sequences related to paternal imprinting of one QTL are combined with sequences relating to genes or favourable alleles of a second QTL. Such a gene construct is favourably used to obtain transgenic animals wherein the second QTL has been equipped with paternal imprinting, as opposed to the inheritance pattern in the native animal from which the second QTL is derived. Such a second QTL can for example be derived from the same chromosome where the parental imprinting region is located, but can also be derived from a different chromosome from the same or even a different species. In the pig, such a second QTL can for example be related to an oestrogen receptor (ESR)-gene (Rothschild et al, PNAS, 93, 201-201, 1996) or a FAT-QTL (Andersson, Science, 263, 1771-1774, 1994) for example derived from an other pig chromosome, such as chromosome 4.

The invention furthermore provides an isolated and/or recombinant nucleic acid or functional fragment derived thereof at least partly corresponding to a QTL of a pig located at a Sus scrofa chromosome 2 mapping at position 2p1.7 wherein said QTL is related to the potential muscle mass and/or fat deposition of said pig and/or wherein said QTL comprises at least a part of a Sus scrofa insulin-like growth factor-2 (IGF2) allele, preferably at least spanning a region between INS and H19, or preferably derived from a domestic pig, such as a Pietrain, Meishan, Duroc, Landrace or Large White, or from a Wild Boar. For example, a genomic *IGF2* clone was isolated by screening a porcine BAC library. FISH analysis with this BAC clone gave a strong consistent signal on the terminal part of chromosome 2p. A polymorphic microsatellite is located in the 3'UTR of *IGF2* in mice (GenBank U71085), humans (GenBank S62623), and horse (GenBank AF020598). The possible presence of a corresponding porcine microsatellite was investigated by direct sequencing of the *IGF2* 3'UTR using the BAC clone.

A complex microsatellite was identified about 800 bp downstream of the stop codon; a sequence comparison revealed that this microsatellite is identical to a previously described anonymous microsatellite, *Swc9*. PCR primers were designed and the microsatellite (*IGF2ms)* was found to be highly polymorphic with three different alleles among the two Wild Boar founders and another two among the eight Large White founders. *IGF2ms* was fully informative in the intercross as the breed of origin as well as the parent of origin could be determined with confidence for each allele in each F_2 animal.

A linkage analysis using the intercross pedigree was carried out with *IGF2ms* and the microsatellites *Sw2443*, *Sw2623*, and *Swr2516*, all from the distal end of 2p[7]. *IGF2* was firmly assigned to 2p by highly significant lod scores (e.g. Z=89.0, θ=0.003 against *Swr2516*). Multipoint analyses, including previously typed chromosome 2 markers, revealed the following order of loci (sex-average map distances in Kosambi cM): *Sw2443/Swr2516*-0.3-*IGF2*-14.9-*Sw2623*-10.3-*Sw256*. No recombinant was observed between *Sw2443* and *Swr2516*, and the suggested proximal location of *IGF2* in relation to these loci is based on a single recombinant giving a lod score support of 0.8 for the reported order. The most distal marker in our previous QTL study, *Sw256*, is located about 25 cM from the distal end of the linkage group.

The invention furthermore provides use of a nucleic acid or functional fragment derived thereof according to the invention in a method according to the invention. In a preferred embodiment, use of a method according to invention is provided to select a breeding animal or animal destined for slaughter for having desired genotypic or potential phenotypic properties. In particular, the invention provides such use wherein said properties are related to muscle mass and/or fat deposition. The QTL as provided by the invention may be

exploited or used to improve for example lean meat content or back-fat thickness by marker assisted selection within populations or by marker assisted introgression of favorable alleles from one population to another. Examples of marker assisted selection using the QTL as provided by the invention are use of marker assisted segregation analysis with linked markers or with markers in disequilibrium to identify functionally distinct QTL alleles. Furthermore, identification of a causative mutation in the QTL is now possible, again leading to identify functionally distinct QTL alleles. Such functionally distinct QTL alleles located at the distal tip of chromosome 2p with large effects on skeletal muscle mass, the size of the heart, and on back-fat thickness are also provided by the invention. The observation of a similar QTL effect in a Large White x Wild Boar as well as in a Piétrain x Large White intercross provides proof of the existence of a series of at least three distinct functional alleles. Moreover, preliminary evidence based on marker assisted segregation analysis points towards residual segregation at this locus within the Piétrain population (data not shown). The occurrence of an allelic series as provided by the invention allows identifying causal polymorphisms which - based on the quantitative nature of the observed effect - are unlikely to be gross gene alterations but rather subtle regulatory mutations. The effects on muscle mass of the three alleles rank in the same order as the breeds in which they are found i.e. Piétrain pigs are more muscular than Large White pigs that in turn have higher lean meat content than Wild Boars. The invention furthermore provides use of the alleles as provided by the invention for within line selection or for marker assisted introgression using linked markers, markers in disequilibrium or alleles comprising causative mutations.

The invention furthermore provides an animal selected by using a method according to the invention.

For example, a pig characterised in being homozygous for an allele in a QTL located at a Sus scrofa chromosome 2 mapping at position 2p1.7 can now be selected and is thus provided by the invention. Since said QTL is related to the potential muscle mass and/or fat deposition of said pig and/or said QTL comprises at least a part of a Sus scrofa insulin-like growth factor-2 (IGF2) allele, it is possible to select promising pigs to be used for breeding or to be slaughtered. In particular an animal according to the invention which is a male is provided. Such a male, or its sperm or an embryo derived thereof can advantageously be used in breeding animals for creating breeding lines or for finally breeding animals destined for slaughter. In a preferred embodiment of such use as provided by the invention, a male, or its sperm, deliberately selected for being homozygous for an allele causing the extreme muscular hyperthrophy and leanness, is used to produce offspring heterozygous for such an allele. Due to said allele's paternal expression, said offspring will also show the favourable traits for example related to muscle mass, even if the parent female has a different genetic background. Moreover, it is now possible to positively select the female(s) for having different traits, for example related to fertility, without having a negative effect on the muscle mass trait that is inherited from the allele from the selected male. For example, earlier such males could occasionally be seen with Piétrain pigs but genetically it was not understood how to most profitably use these traits in breeding programmes.

Furthermore, the invention provides a transgenic animal, sperm and an embryo derived thereof, comprising a synthetic parentally imprinted QTL or functional fragment thereof as provided by the invention, i.e. it is provided by the invention to introduce a favourable allele of for example the oestrogen receptor locus related to increased litter size of an animal in a maternally imprinted region or to introduce a favourable fat-related allele or muscle

mass-related allele in a paternally imprinted region, or vice versa.

The invention is further explained in the experimental part without limiting the invention.

Experimental part.

Example 1: Wild Boar x Large White intercrosses

Methods

Isolation of an *IGF2* BAC clone and fluorescent *in situ* hybridization (FISH). *IGF2* primers (F:5'-GGCAAGTTCTTCCGCTAATGA-3' and R:5'-GCACCGCAGAATTACGACAA-3') for PCR amplification of a part of the last exon and 3'UTR were designed on the basis of a porcine IGF2 cDNA sequence (GenBank X56094). The primers were used to screen a porcine BAC library and the clone 253G10 was isolated. Crude BAC DNA was prepared as described[24]. The BAC DNA was linearized with *Eco*RV and purified with QIAEXII (QIAGEN GmbH, Germany). The clone was labeled with biotin-14-dATP using the GIBCO-BRL Bionick labeling system (BRL18246-015). Porcine metaphase chromosomes were obtained from pokeweed (Seromed) stimulated lymphocytes using standard techniques. The slides were aged for two days at room temperature and then kept at -20°C until use. FISH analysis was carried out as previously described[25]. The final concentration of the probe in the hybridization mix was 10 ng/µl. Repetitive sequences were suppressed with standard concentrations of porcine genomic DNA. After post-hybridization washing, the biotinylated probe was detected with two layers of avidin-FITC (Vector A-2011). The chromosomes were counterstained with 0.3 mg/ml DAPI (4,6-Diamino-2-phenylindole; Sigma D9542), which produced a G-banding like pattern. No posthybridization banding was needed,

since chromosome 2 is easily recognized without banding. A total of 20 metaphase spreads were examined under an Olympus BX-60 fluorescence microscope connected to an IMAC-CCD S30 video camera and equipped with an ISIS 1.65 (Metasystems) software.

Sequence, microsatellite, and linkage analysis.

About two µg of linearized and purified BAC DNA was used for direct sequencing with 20 pmoles of primers and BigDye Terminator chemistry (Perkin Elmer, USA). DNA sequencing was done from the 3' end of the last exon towards the 3' end of the UTR until a microsatellite was detected. A primer set (F:5'-GTTTCTCCTGTACCCACACGCATCCC-3' and R:5'-Fluorescein- CTACAAGCTGGGCTCAGGG-3') was designed for the amplification of the *IGF2* microsatellite which is about 250 bp long and located approximately 800 bp downstream from the stop codon. The microsatellite was PCR amplified using fluorescently labeled primers and the genotyping was carried out using an ABI377 sequencer and the GeneScan/Genotyper softwares (Perkin Elmer, USA). Two-point and multipoint linkage analysis were done with the Cri-Map software[26].

Animals and phenotypic data.

The intercross pedigree comprised two European Wild Boar males and eight Large White females, 4 F_1 males and 22 F_1 females, and 200 F_2 progeny[1]. The F_2 animals were sacrificed at a live weight of at least 80 kg or at a maximum age of 190 days. Phenotypic data on birth weight, growth, fat deposition, body composition, weight of internal organs, and meat quality were collected; a detailed description of the phenotypic traits are

provided by Andersson *et al.*[1] and Andersson-Eklund *et al.*[4]

Statistical analysis.

Interval mapping for the presence of QTL were carried out with a least squares method developed for the analysis of crosses between outbred lines[27]. The method is based on the assumption that the two divergent lines are fixed for alternative QTL alleles. There are four possible genotypes in the F_2 generation as regards the grandparental origin of the alleles at each locus. This makes it possible to fit three effects: additive, dominance, and imprinting[2]. The latter is estimated as the difference between the two types of heterozygotes, the one receiving the Wild Boar allele through an F_1 sire and the one receiving it from an F_1 dam. An F-ratio was calculated using this model (with 3 d.f.) versus a reduced model without a QTL effect for each cM of chromosome 2. The most likely position of a QTL was obtained as the location giving the highest F-ratio. Genome-wise significance thresholds were obtained empirically by a permutation test[28] as described[2]. The QTL model including an imprinting effect was compared with a model without imprinting (with 1 d.f.) to test whether the imprinting effect was significant.

The statistical models also included the fixed effects and covariates that were relevant for the respective traits; see Andersson-Eklund *et al.*[4] for a more detailed description of the statistical models used. Family was included to account for background genetic effects and maternal effects. Carcass weight was included as a covariate to discern QTL effects on correlated traits, which means that all results concerning body composition were compared at equal weights. Least-squares

means for each genotype class at the *IGF2* locus were estimated with a single point analysis using Procedure GLM of SAS[29]; the model included the same fixed effects and covariates as used in the interval mapping analyses. The QTL shows a clear parent of origin-specific expression and the map position coincides with that of the insulin-like growth factor II gene (*IGF2*), indicating *IGF2* as the causative gene. A highly significant segregation distortion (excess of Wild Boar-derived alleles) was also observed at this locus. The results demonstrate an important effect of the *IGF2* region on postnatal development and it is possible that the presence of a paternally expressed *IGF2*-linked QTL in humans and in rodent model organisms has so far been overlooked due to experimental design or statistical treatment of data. The study has also important implications for quantitative genetics theory and practical pig breeding.

IGF2 was identified as a positional candidate gene for this QTL due to the observed similarity between pig chromosome 2p and human chromosome 11p. A genomic *IGF2* clone was isolated by screening a porcine BAC library. FISH analysis with this BAC clone gave a strong consistent signal on the terminal part of chromosome 2p (Fig. 1). A polymorphic microsatellite is located in the 3'UTR of *IGF2* in mice (GenBank U71085), humans (GenBank S62623), and horse (GenBank AF020598). The possible presence of a corresponding porcine microsatellite was investigated by direct sequencing of the *IGF2* 3'UTR using the BAC clone. A complex microsatellite was identified about 800 bp downstream of the stop codon; a sequence comparison revealed that this microsatellite is identical to a previously described anonymous microsatellite, *Swc9*[6]. PCR primers were designed and the microsatellite (*IGF2ms*) was found to be highly polymorphic with three

different alleles among the two Wild Boar founders and another two among the eight Large White founders. *IGF2ms* was fully informative in the intercross as the breed of origin as well as the parent of origin could be determined with confidence for each allele in each F_2 animal.

A linkage analysis using the intercross pedigree was carried out with *IGF2ms* and the microsatellites *Sw2443*, *Sw2623*, and *Swr2516*, all from the distal end of 2p[7]. *IGF2* was firmly assigned to 2p by highly significant lod scores (e.g. Z=89.0, θ=0.003 against *Swr2516*). Multipoint analyses, including previously typed chromosome 2 markers[8], revealed the following order of loci (sex-average map distances in Kosambi cM): *Sw2443/Swr2516*-0.3-*IGF2*-14.9-*Sw2623*-10.3-*Sw256*. No recombinant was observed between *Sw2443* and *Swr2516*, and the suggested proximal location of *IGF2* in relation to these loci is based on a single recombinant giving a lod score support of 0.8 for the reported order. The most distal marker in our previous QTL study, *Sw256*, is located about 25 cM from the distal end of the linkage group.

QTL analyses of body composition, fatness, meat quality, and growth traits were carried out with the new chromosome 2 map using a statistical model testing for the possible presence of an imprinting effect as expected for *IGF2*. Clear evidence for a paternally expressed QTL located at the very distal tip of 2p was obtained (Fig. 2; Table 1). The QTL had very large effects on lean meat content in ham and explained an astonishing 30% of the residual phenotypic variance in the F_2 population. Large effects on the area of the longissumus dorsi muscle, on the weight of the heart, and on back-fat thickness (subcutaneous fat) were also noted. A moderate effect on one meat quality trait, reflectance value, was indicated. The QTL had no significant effect on abdominal fat, birth

weight, growth, weight of liver, kidney, or spleen (data not shown). The Large White allele at this QTL was associated with larger muscle mass and reduced back-fat thickness consistent with the difference between this breed and the Wild Boar population. The strong imprinting effect observed for all affected traits strongly suggests a single causative locus. The pleiotropic effects on skeletal muscle mass and the size of the heart appear adaptive from a physiological point of view as a larger muscle mass requires a larger cardiac output. The clear paternal expression of this QTL is illustrated by the least squares means which fall into two classes following the population origin of the paternally inherited allele (Table 1). It is worth noticing though that there was a non-significant trend towards less extreme values for the two heterozygous classes, in particular for the estimated effect on the area of longissimus dorsi. This may be due to chance, but could have a biological explanation, e.g. that there is some expression of the maternally inherited allele or that there is a linked, non-imprinted QTL with minor effects on the traits in question.

The *IGF2*-linked QTL and the *FAT1* QTL on chromosome 4 1, 9 are by far the two loci with the largest effect on body composition and fatness segregating in this Wild Boar intercross. The *IGF2* QTL controls primarily muscle mass whereas *FAT1* has major effects on fat deposition including abdominal fat, a trait that was not affected by the *IGF2* QTL (Fig. 2). No significant interaction between the two loci was indicated and they control a very large proportion of the residual phenotypic variance in the F_2 generation. A model including both QTLs explains 33.1% of the variance for percentage lean meat in ham, 31.3% for the percentage of lean meat plus bone in back, and 26.2% for average back fat depth (compare with a model including only chromosome 2 effects, Table 1). The two QTLs must have played a major role in the response during

selection for lean growth and muscle mass in the Large White domestic pig.

A highly significant segregation distortion was observed in the *IGF2* region (excess of Wild Boar-derived alleles) as shown in Table 1 ($\chi 2=11.7$, d.f.=2; P=0.003). The frequency of Wild Boar-derived *IGF2* alleles was 59% in contrast to the expected 50% and there was twice as many "Wild Boar" as "Large White" homozygotes. This deviation was observed with all three loci at the distal tip and is thus not due to typing errors. The effect was also observed with other loci but the degree of distortion decreased as a function of the distance to the distal tip of the chromosome. Blood samples for DNA preparation were collected at 12 weeks of age and we are convinced that the deviation from expected Mendelian ratios was present at birth as the number of animals lost prior to blood sampling was not sufficient to cause a deviation of this magnitude. No other of the more than 250 loci analyzed in this pedigree show such a marked segregation distortion (L. Andersson, unpublished). The segregation distortion did not show an imprinting effect, as the frequencies of the two reciprocal types of heterozygotes were identical (Table 1). This does not exclude the possibility that the QTL effects and the segregation distortion are controlled by the same locus. The segregation distortion maybe due to meiotic drive favoring the paternally expressed allele during gametogenesis, as the F_1 parents were all sired by Wild Boar males. Another possibility is that the segregation distortion may be due to codominant expression of the maternal and paternal allele in some tissues and/or during a critical period of embryo development. Biallelic *IGF2* expression has been reported to occur to some extent during human development[10, 11] and interestingly a strong influence of the parental species background on *IGF2* expression was recently found in a cross between *Mus*

musculus and *Mus spretus*[12]. It is also interesting that a VNTR polymorphism at the insulin gene, which is very closely linked to *IGF2*, is associated with size at birth in humans[13]. It is possible that the *IGF2*-linked QTL in pigs has a minor effect on birth weight but in our data it was far from significant (Fig. 2) and there was no indication of an imprinting effect.

This study is an advance in the general knowledge concerning the biological importance of the *IGF2* locus. The important role of *IGF2* for prenatal development is well-documented from knock-out mice[14] as well as from its causative role in the human Beckwith-Wiedemann syndrome[15]. This study demonstrates an important role for the *IGF2*-region also for postnatal development. It should be stressed that our intercross between outbred populations is particularly powerful to detect QTL with a parent of origin-specific effect on a multifactorial trait. This is because multiple alleles (or haplotypes) are segregating and we could deduce whether a heterozygous F_2 animal received the Wild Boar allele from the F_1 male or female. It is quite possible that the segregation of a paternally expressed *IGF2*-linked QTL affecting a trait like obesity has been overlooked in human studies or in intercrosses between inbred rodent populations because of experimental design or statistical treatment of data. An imprinting effect cannot be detected in an intercross between two inbred lines as only two alleles are segregating at each locus. Our result has therefore significant bearings on the future analysis of the association between genetic polymorphism in the *insulin-IGF2* region and Type I diabetes[16], obesity[17], and variation in birth weight[13] in humans, as well as for the genetic dissection of complex traits using inbred rodent models. A major impetus for generating an intercross between the domestic pig and its

wild ancestor was to explore the possibilities to map and identify major loci that have responded to selection. We have now showed that two single QTLs on chromosome 2 (this study) and 4[1, 2] explain as much as one third of the phenotypic variance for lean meat content in the F_2 generation. This is a gross deviation from the underlying assumption in the classical infinitesimal model in quantitative genetics theory namely that quantitative traits are controlled by an infinite number of loci each with an infinitesimal effect. If a large proportion of the genetic difference between two divergent populations (e.g. Wild Boar and Large White) is controlled by a few loci, one would assume that selection would quickly fix QTL alleles with large effects leading to a selection plateau. However, this is not the experience in animal breeding programs or selection experiments where good persistent long-term selection responses are generally obtained, provided that the effective population size is reasonably large[18]. A possible explanation for this paradox is that QTL alleles controlling a large proportion of genetic differences between two populations may be due to several consecutive mutations; this may be mutations in the same gene or at several closely linked genes affecting the same trait. It has been argued that new mutations contribute substantially to long-term selection responses[19], but the genomic distribution of such mutations are unknown.

The search for a single causative mutation is the paradigm as regards the analysis of genetic defects in mice and monogenic disorders in humans. We propose that this may not be the case for loci that have been under selection for a large number of generations in domestic animals, crops, or natural populations. This hypothesis predicts the presence of multiple alleles at major QTL. It gains some support from our recent characterization of porcine coat color variation. We have found that both the

alleles for dominant white color and for black-spotting differ from the corresponding wild-type alleles by at least two consecutive mutations with phenotypic effects at the *KIT* and *MC1R* loci, respectively[20, 21]. In this context it is highly interesting that in the accompanying example we have identified a third allele at the *IGF2*-linked QTL. The effects on muscle mass of the three alleles rank in the same order as the breeds in which they are found i.e. Piétrain pigs are more muscular than Large White pigs that in turn have higher lean meat content than Wild Boars.

There are good reasons to decide that *IGF2* is the causative gene for the now reported QTL. Firstly, there is a perfect agreement in map localization (Fig. 2). Secondly, it has been shown that *IGF2* is paternally expressed in mice, humans, and now in pigs, like the QTL. There are several other imprinted genes in the near vicinity of *IGF2* in mice and humans (*Mash2, INS2, H19, KVLQT1, TAPA1/CD81*, and *CDKN1C/p57^{KIP2}*) but only *IGF2* is paternally expressed in adult tissues[22]. We believe that this locus provides a unique opportunity for molecular characterization of a QTL. The clear paternal expression can be used to exclude genes that do not show this mode of inheritance. Moreover, the presence of an allelic series should facilitate the difficult distinction between causative mutations and linked neutral polymorphism. We have already shown that there is no difference in coding sequence between *IGF2* alleles from Piétrain and Large White pigs suggesting that the causative mutations occur in regulatory sequences. An obvious step is to sequence the entire *IGF2* gene and its multiple promoters from the three populations. The recent report that a VNTR polymorphism in the promoter region of the insulin (*INS*) gene affects *IGF2* expression[23] suggests

that the causative mutations may be at a considerable distance from the *IGF2* coding sequence.

The results have several important implications for the pig breeding industry. They show that genetic imprinting is not an esoteric academic question but need to be considered in practical breeding programs. The detection of three different alleles in Wild Boar, Large White, and Piétrain populations indicates that further alleles at the *IGF2*-linked QTL segregate within commercial populations. The paternal expression of the QTL facilitates its detection using large paternal half-sib families as the female contribution can be ignored. The QTL is exploited to improve lean meat content by marker assisted selection within populations or by marker assisted introgression of favorable alleles from one population to another.

Example 2: Piétrain x Large White intercrosses

Methods

Pedigree material: The pedigree material utilized to map QTL was selected from a previously described Piétrain x Large White F2 pedigree comprising > 1,800 individuals[6,7]. To assemble this F2 material, 27 Piétrain boars were mated to 20 Large White sows to generate an F1 generation comprising 456 individuals. 31 F1 boars were mated to unrelated 82 F1 sows from 1984 to 1989, yielding a total of 1862 F2 offspring. F1 boars were mated on average to 7 females, and F1 sows to an average of 2,7 males. Average offspring per boar were 60 and per sow 23.

Phenotypic information: (i) Data collection: A total of 21 distinct phenotypes were recorded in the F2 generation[6,7]. These included:

- five growth traits: birth weight (g), weaning weight (Kg), grower weight (Kg), finisher weight (Kg) and average daily gain (ADG; Kg/day; grower to finsher period);
- two body proportion measurements: carcass length (cm); and a conformation score (0 to 10 scale; ref.6);
- ten measurements of carcass composition obtained by dissection of the chilled carcasses 24 hours after slaughter. These include measurements of muscularity: % ham (weight hams/carcass weight), % loin (weight loin/carcass weight), % shoulder (weight shoulder/carcass weight), % lean cuts (% ham + %loin + % shoulder); and measurements of fatness: average back-fat thickness (BFT; cm), % backfat (weight backfat/carcass weight), % belly (weight belly/carcass weight), % leaf fat (weight leaf fat/carcass weight), % jowl (weight jowl/carcass weight), and "% fat cuts" (% backfat + % belly + % leaft fat + % jowl).
- four meat quality measurements: pH_{LD1} (*Longissimus dorsi* 1

hour after slaughter), pH $_{LD24}$ (*Longissimus dorsi* 24 hours after slaughter), pH $_{G1}$ (*Gracilis* 1 hour after slaughter) and pH $_{G24}$ (*Gracilis* 24 hours after slaughter). *(ii) Data processing:* Individual phenotypes were preadjusted for fixed effects (sire, dam, CRC genotype, sex, year-season, parity) and covariates (litter size, birth weight, weaning weight, grower weight, finisher weight) that proved to significantly affect the corresponding trait. Variables included in the model were selected by stepwise regression.

Marker genotyping: Primer pairs utilized for PCR amplification of microsatellite markers are as described[19]. Marker genotyping was performed as previously described[20]. Genotypes at the *CRC* and *MyoD* loci were determined using conventional methods as described[1,12]. The LAR test for the Igf2 SNP was developed according to Baron et al.[21] using a primer pair for PCR amplification (5'-CCCCTGAACTTGAGGACGAGCAGCC-3';5'-ATCGCTGTGGGCTGGGTGGGCTGCC-3') and a set of three primers for the LAR step (5'-FAM-CGCCCCAGCTGCCCCCCAG-3'; 5'-HEX-CGCCCCAGCTGCCCCCCAA-3'; 5'-CCTGAGCTGCAGCAGGCCAG-3').

Map construction: Marker maps were constructed using the TWOPOINT, BUILD and CHROMPIC options of the CRIMAP package[22]. To allow utilisation of this package, full-sib families related via the boar or sow were disconnected and treated independently. By doing so, some potentially usable information was neglected, yielding, however, unbiased estimates of recombination rates.

QTL mapping: (i) Mapping Mendelian QTL: Conventional QTL mapping was performed using a multipoint maximum likelihood method. The applied model assumed one segregating QTL per

chromosome, and fixation of alternate QTL alleles in the respective parental lines, Piétrain (P) and Large White (LW). A specific analysis program had to be developed to account for the missing genotypes of the parental generation, resulting in the fact that the parental origin of the F1 chromosomes could not be determined. Using a typical "interval mapping" strategy, an hypothetical QTL was moved along the marker map using user-defined steps. At each position, the likelihood *(L)* of the pedigree data was computed as:

$$L = \sum_{\varphi=1}^{2^r} \prod_{i=1}^{n} \sum_{G=1}^{4} (P(G|M_i,\theta,\varphi)P(y_i|G))$$

P or right chromosme *P*), there is a total of 2^r combinations for *r* F1 parents.

$\prod_{i=1}^{n}$ *n* F2

$\sum_{G=1}^{4}$ *i*th F2 offspring, over the four possible QTL genotypes: *P/P, P/LW, LW/P* and *LW/LW*

$P(G|M_i,\theta,\varphi)M_i$: the marker genotype of the *i*th F2 offspring and its F1 parents, (ii) : the vector of recombination rates between adjacent markers and between the hypothetical QTL and its flanking markers, and (iii)θ the considered marker-QTL phase combination of the F1 parents.

Recombination rates and marker linkage phase of F1 parents are assumed to be known when computing this probability. Both were determined using CRIMAP in the map construction phase (see above).

$P(y_i|G)$ y_i) of offspring *i*, given the QTL genotype under consideration. This probability is computed from the normal density function:

$$P(y_i|G) = \frac{1}{\sqrt{2\pi}\sigma} e^{\frac{-(y_i-\mu_G)^2}{2\sigma^2}}$$

$_G$ is the phenotypic mean of the considered QTL genotype (PP, PL, LP or LL) and σ^2 the residual variance σ^2 was considered to be the same for the four QTL genotypic classes.

The values of μ_{PP}, $\mu_{PL}=\mu_{LP}$, μ_{LL} and σ^2 maximizing L were determined using the GEMINI optimisation routine[23].

The likelihood obtained under this alternative H_1 hypothesis was compared with the likelihood obtained under the null hypothesis H_0 of no QTL, in which the phenotypic means of the four QTL genotypic classes were forced to be identical. The difference between the logarithms of the corresponding likelihoods yields a lodscore measuring the evidence in favour of a QTL at the corresponding map position.

*(ii) Significance thresholds:*Following Lander & Botstein[24], lodscore thresholds *(T)* associated with a chosen genome-wise significance level, were computed such that:

$$\alpha = (C + 9.21GT)\chi_2^2(4.6T)$$

C corresponds to the number of chromosomes (= 19), *G* corresponds to the length of the genome in Morgans (= 29), and $\chi_2^2(4.6T)$ denotes one minus the cumulative distribution function of the chi-squared distribution with 2 d.f. Single point 2ln(LR) were assumed to be distributed as a chi-squared distribution with two degrees of freedom, as we were fitting both an additive and dominance component. To account for the fact that we were analysing multiple traits, significance levels were adjusted by applying a Bonferoni correction corresponding to the effective number of independent traits that were analyzed. This effective number was estimated at 16 following the approach described by Spelman et al.[25].

Altogether, this allowed us to set the lodscore threshold associated with an experiment-wise significance level of 5%

at 5.8. When attempting to confirm the identified QTL in an independent sample, the same approach was used, however, setting C at 1, G at 25cM and correcting for the analysis of 4.5 independent traits (as only six traits were analyzed in this sample). This yielded a lodscore threshold associated with a Type I error of 5% of 2.

(iii). Testing for an imprinted QTL: To test for an imprinted QTL, we assumed that only the QTL alleles transmitted by the parent of a given sex would have an effect on phenotype, the QTL alleles transmitted by the other parent being "neutral". The likelihood of the pedigree data under this hypothesis was computed using equation 1. To compute $P(y_i \mid G)$, however, the phenotypic means of the four QTL genotypes were set at $\mu_{PP} = \mu_{PL} = \mu_P$ and $\mu_{LP} = \mu_{LL} = \mu_L$ to test for a QTL for which the paternal allele only is expressed, and $\mu_{PP} = \mu_{LP} = \mu_P$ and $\mu_{PL} = \mu_{LL} = \mu_L$ to test for a QTL for which the maternal allele only is expressed. It is assumed in this notation that the first subscript refers to the paternal allele, the second subscript to the maternal allele. H_0 was defined as the null-hypothesis of no QTL, H_1 testing the presence of a Mendelian QTL; H_2 testing the presence of a paternally expressed QTL, and H_3 testing the presence of a maternally expressed QTL.

RT-PCR: Total RNA was extracted from skeletal muscle according to Chirgwin et al.[26]. RT-PCR was performed using the Gene-Amp RNA PCR Kit (Perkin-Elmer) The PCR products were purified using QiaQuick PCR Purification kit (Qiagen) and sequenced using Dye terminator Cycle Sequencing Ready Reaction (Perkin Elmer) and an ABI373 automatic sequencer.

In example 2 we report the identification of a QTL with major effect on muscle mass and fat deposition mapping to porcine 2p1.7 The QTL shows clear evidence for parental imprinting strongly suggesting the involvement of the *Igf2* locus.

A Piétrain X Large White intercross comprising 1125 F_2 offspring was generated as described[6,7]. The Large White and Piétrain parental breeds differ for a number of economically important phenotypes. Piétrains are famed for their exceptional muscularity and leannes [8] (Figure 2), while Large Whites show superior growth performance. Twenty-one distinct phenotypes measuring (i) growth performance (5), (ii) muscularity (6), (iii) fat deposition (6), and (iv) meat quality (4), were recorded on all F_2 offspring.

In order to map QTL underlying the genetic differences between these breeds, we undertook a whole genome scan using microsatellite markers on an initial sample of 677 F_2 individuals. Analysis of pig chromosome 2 using a ML multipoint algorithm, revealed highly significant lodscores (up to 20) for six of the 12 phenotypes measuring muscularity and fat deposition at the distal end of the short arm of chromosome 2 (Figure 3a). Positive lodscores were obtained for the remaining six phenotypes, however, not reaching the genome-wise significance threshold (= 5%). To confirm this finding, the remaining sample of 355 F_2 offspring was genotyped for the five most distal 2p markers and QTL analysis performed for the traits yielding the highest lodscores in the first analysis. Lodscores ranged from 2.1 to 7.7, clearly confirming the presence of a major QTL in this region. Table 2 reports the corresponding ML estimates for the three genotypic means as well as the corresponding residual variance.

Bidirectional chromosome painting establishes a correspondence between SSC2p and HSA11pter-q13[9,10]. At least

two serious candidate genes map to this region in man: the myogenic basic helix-loop-helix factor, *MyoD*, maps to HSA11p15.4, while *Igf2* maps to HSA11p15.5 *MyoD* is a well known key regulator of myogenesis and is one of the first myogenic markers to be switched on during development[11]. A previously described amplified sequence polymorphism in the porcine *MyoD* gene[12] proved to segregate in our F_2 material, which was entirely genotyped for this marker. Linkage analysis positioned the *MyoD* gene in the SW240-SW776 (odds > 1000) interval, therefore well outside the lod-2 drop off support interval for the QTL (figure 1). *Igf2* is known to enhance both proliferation and differentiation of myoblasts *in vitro*[13] and to cause a muscular hypertrophy when overexpressed *in vivo*. Based on a published porcine adult liver cDNA sequence[14], we designed primer pairs allowing us to amplify the entire *Igf2* coding sequence with 222 bp of leader and 280 bp of trailor sequence from adult skeletal muscle cDNA. Piétrain and Large White RT-PCR products were sequenced indicating that the coding sequences was identical in both breeds and with the published sequence. However, a G A transition was found in the leader sequence corresponding to exon 2 in man (Figure 4). We developed a screening test for this single nucleotide polymorphism (SNP) based on the ligation amplification reaction (LAR), allowing us to genotype our pedigree material. Based on these data, *Igf2* was shown to colocalize with the SWC9 microsatellite marker (= 0%), therefore located at approximately 2 centimorgan from the most likely position of the QTL and well within the 95% support interval for the QTL (figure 1). Subsequent sequence analysis demonstrated that the microsatellite marker SWC9 is actually located within the 3' UTR of the *Igf2* gene. Combined with available comparative mapping data for the PGA and FSH loci, these results suggest the occurrence of an interstitial

inversion of a chromosome segment containing *MyoD*, but not *Igf2* which has remained telomeric in both species.

Igf2 therefore appeared as a strong positional allele having the observed QTL effect. In man and mouse, *Igf2* is known to be imprinted and to be expressed exclusively from the paternal allele in several tissues[15]. Analysis of skeletal muscle cDNA from pigs heterozygous for the SNP and/or SWC9, shows that the same imprinting holds in this tissue in the pig as well (Figure 4). Therefore if *Igf2* were responsible for the observed effect, and knowing that only the paternal *Igf2* allele is expressed, one can predict that (i) the paternal allele transmitted by F1 boars (P or LW) would have an effect on phenotype of F2 offspring, (ii) the maternal allele transmitted by F1 sows (P or LW) would have no effect on phenotype of F2 offspring, and (iii) the likelihood of the data would be superior under a model of a bimodal (1:1) F2 population sorted by inherited paternal allele when compared to a conventional "Mendelian" model of a trimodal (1:2:1) F2 population. The QTL mapping programs were adapted in order to allow testing of the corresponding hypotheses. H_0 was defined as the null-hypothesis of no QTL, H_1 as testing for the presence of a Mendelian QTL, H_2 as testing for the presence of a paternally expressed QTL, and H_3 as testing for the presence of a maternally expressed QTL.

Figure 3 summarizes the obtained results. Figure 3a, 3b and 3c respectively show the lodscore curves corresponding to $\log_{10}(H_2/H_0)$, $\log_{10}(H_3/H_0)$ and $\log_{10}(H_2/H_1)$. It can be seen that very significant lodscores are obtained when testing for the presence of a paternally expressed QTL, while there is no evidence at all for the segregation of a QTL when studying the chromosomes transmitted by the sows. Also, the hypothesis of a paternally expressed QTL is significantly more likely ($\log_{10}(H_2/H_1) > 3$) than the hypothesis of a "Mendelian" QTL

for all examined traits. The fact that the same tendency is observed for all traits indicates that it is likely the same imprinted gene that is responsible for the effects observed on the different traits. Table 2 reports the ML phenotypic means for the F2 offspring sorted by inherited paternal QTL allele. Note that when performing the analysis under a model of a mendelian QTL, the Piétrain and Large White QTL alleles appeared to behave in an additive fashion, the heterozygous genotype exhibiting a phenotypic mean corresponding exactly to the midpoint between the two homzygous genotypes. This is exactly what one would predict when dealing with an imprinted QTL as halve of the heterozygous offspring are expected to have inherited the P allele from their sire, the other halve the LW allele.

These data therefore confirmed our hypothesis of the involvement of an imprinted gene expressed exclusively from the paternal allele. The fact that the identified chromosomal segment coincides precisely with an imprinted domain documented in man and mice strongly implicates the orthologous region in pigs. At least seven imprinted genes mapping to this domain have been documented (*Igf2*, *Ins2*, *H19*, *Mash2*, *p57^{KIP2}*, *K$_v$LQTL1* and *TDAG51*) (ref. 15 and Andrew Feinberg, personal communication). Amongst these, only *Igf2* and *Ins2* are paternally expressed. While we cannot exclude that the observed QTL effect is due to an as of yet unidentified imprinted gene in this region, its reported effects on myogenesis *in vitro* and *in vivo*[13] strongly implicate *Igf2*. Particularly the muscular hypertrophy observed in transgenic mice overexpressing *Igf2* from a muscle specific promotor are in support of this hypothesis (Nadia Rosenthal, personal communication. Note that allelic variants of the *INS* VNTR have recently been shown to be associated

with size at birth in man[16], and that the same VNTR has been shown to affect the level of *Igf2* expression[17].

The observation of the same QTL effect in a Large White x Wild Boar intercross indicates the existence of a series of at least three distinct functional alleles. Moreover, preliminary evidence based on marker assisted segregation analysis points towards residual segregation at this locus within the Piétrain population (data not shown). The occurrence of an allelic series might be invaluable in identifying the causal polymorphisms which - based on the quantitatve nature of the observed effect - are unlikely to be gross gene alterations but rather subtle regulatory mutations.

The effects of the identified QTL on muscle mass and fat deposition are truly major, being of the same magnitude of those reported for the *CRC* locus[6,7] though apparently without the associated deleterious effects on meat quality. We estimate that both loci jointly explain close to 50% of the Piétrain versus Large White breed difference for muscularity and leanness. Understanding the parent-of-origin effect characterizing this locus will allow for its optimal use in breeding programs. Indeed, today half of the offspring from commercially popular Piétrain x Large White crossbred boars inherit the unfavourable Large White allele causing considerable loss.

The QTL described in this work is the second example of a gene affecting muscle development in livestock species that exhibits a non-mendelian inheritance pattern. Indeed, we have previously shown that the callipyge locus (related to the qualitative trait wherein muscles are doubled) is characterized by polar overdominance in which only the heterozygous individuals that inherit the CLPG mutation from their sire express the double-muscling phenotype[5]. This

demonstrates that parent-of-origin effects affecting genes underlying production traits in livestock might be relatively common.

Legends to the figures

Fig. 1: Test statistic curves obtained in QTL analyses of chromosome 2 in a Wild Boar/Large White intercross. The graph plots the F ratio testing the hypothesis of a single QTL at a given position along the chromosome for the traits indicated. The marker map with the distances between markers in Kosambi centiMorgan is given on the X-axis. The horizontal lines represent genome-wise significant ($P<0.05$) and suggestive levels for the trait lean meat in ham; similar significance thresholds were obtained for the other traits.

Figure 2: Piétrain pig with characteristic muscular hypertrophy.

Figure 3: Lodscore curves obtained in a Piétrain x Large White intercross for six phenotypes measuring muscle mass and fat deposition on pig chromosome 2. The most likely positions of the *Igf2* and *MyoD* genes determined by linkage analysis with respect to the microsatellite marker map are shown. H_0 was defined as the null-hypothesis of no QTL, H_1 as testing for the presence of a Mendelian QTL, H_2 as testing for the presence of a paternally expressed QTL, and H_3 as testing for the presence of a maternally expressed QTL. 3a: $\log_{10}(H_1/H_0)$, 3b:$\log_{10}(H_2/H_0)$, 3c: $\log_{10}(H_3/H_0)$

Figure 4: A. Structure of the human *Igf2* gene according to ref. 17, with aligned porcine adult liver cDNA sequence as reported in ref. 16. The position of the *nt241(G-A)* transition and *Swc9* microsatellite are shown. B. The corresponding markers were used to demonstrate the monoallelic (paternal) expression of *Igf2* in skeletal muscle

and liver of 10-week old fetuses. PCR amplification of the *nt421(G-A)* polymorphism and *Swc9* microsatellite from genomic DNA clearly shows the heterozygosity of the fetus, while only the paternal allele is detected in liver cDNA (*nt421(G-A)* and *Swc9*) and muscle cDNA (*Swc9*). The absence of RT-PCR product for *nt421(G-A)* from in fetal muscle points towards the absence of mRNA including exon 2 in this tissue. Parental origin of the foetal alleles was determined from the genotypes of sire and dam (data not shown).

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Table 1 Summary of QTL analysis for pig chromosome 2 in a Wild Boar/Large White intercross[1]

Trait	F ratio[2]		Map position[3]	Percent of F_2 variance[4]	Least squares means[5]			
	QTL	*Imprinting*			WP/WM	WP/LM	LP/WM	
LP/LM					n=62	n=43	n=43	n=30
Body composition traits								
Lean meat in ham, %	24.4***	19.1***	0	30.6	63.6[a]	64.2[a]	66.4[b]	67.3[b]
Lean meat mass in ham, kg	18.1***	16.8***	1	24.3	4.69[a]	4.72[a]	4.94[b]	5.02[b]
Lean meat + bone in back, %	12.2**	9.6**	0	17.4	66.3[a]	66.7[a]	69.3[b]	70.8[b]
Longissimus muscle area, cm^2	10.3**	4.8*	1	15.4	31.9[a]	33.0[a]	34.5[b]	35.2[b]
Fatness traits								
Average back fat depth, mm	7.1*	8.7**	0	10.4	27.2[a]	27.7[a]	25.5[b]	24.7[b]
Weight of internal organs								
Heart, gram	9.7**	11.4***	0	14.4	226[a]	225[a]	238[b]	244[b]
Meat quality traits								
Reflectance value, EEL	5.7	6.1*	1	8.1	18.6[a]	18.4[a]	21.8[b]	19.7[a]

*P<0.05; **P<0.01; ***P<0.001

Table 1, continued

[1]Only the traits for which the QTL peak was in the *IGF2* region (0-10 cM) and the test statistic reached the nominal significance threshold of F=3.9 are included.

[2]"*QTL*" is the test statistic for the presence of a QTL under a genetic model with additive, dominance, and imprinting effects (3 d.f.) while "Imprinting" is the test statistic for the presence of an imprinting effect (1 d.f.), both obtained at the position of the QTL peak. Genome-wise significance thresholds, estimated by permutation, were used for the QTL test while nominal significance thresholds were used for the Imprinting test.

[3]In cM from the distal end of 2p; *IGF2* is located at 0.3 cM.

[4]The reduction in the residual variance of the F_2 population effected by inclusion of an imprinted QTL at the given position.

[5]Means and standard errors estimated at the *IGF2* locus by classifying the genotypes according to the population and parent of origin of each allele. *W* and *L* represent alleles derived from the Wild Boar and Large White founders, respectively; superscript *P* and *M* represent a paternal and maternal origin, respectively. Figures with different letters (superscript a or b) are significantly different at least at the 5% level, most of them are different at the 1% or 0.1% level.

Table 2 Maximum likelihood phenotypic means for the different F2 genotypes estimated under (i) a model of a mendelian QTL, and (ii) a model assuming an imprinted QTL.

Traits	Mendelian QTL				Imprinted QTL		
	$\mu_{LW/LW}$	$\mu_{LW/P}$	$\mu_{P/P}$	R	$\mu_{PAT/LW}$	$\mu_{PAT/P}$	R
BFT (cm)	2.98	2.84	2.64	0.27	2.94	2.70	0.27
% ham	21.10	21.56	22.15	0.83	21.23	21.95	0.83
% loin	24.96	25.53	26.46	0.91	25.12	26.14	0.93
% lean cuts	65.02	65.96	67.60	1.65	65.23	67.05	1.67
% backfat	6.56	6.02	5.33	0.85	6.43	5.56	0.85
% fat cuts	28.92	27.68	26.66	1.46	28.54	26.99	1.49

CLAIMS

1. A method for selecting a domestic animal for having desired genotypic properties comprising testing said animal for the presence of a parentally imprinted quantitative trait locus (QTL).

2. A method according to claim 1 further comprising testing a nucleic acid sample from said animal for the presence of a parentally imprinted quantitative trait locus (QTL).

3. A method according to claim 1 or 2 wherein in the pig said QTL is located at chromosome 2.

4. A method according to claim 2 or 3 wherein said QTL is mapping at around position 2p1.7.

5. A method according to claim 1 to 4 wherein said QTL is related to the potential muscle mass and/or fat deposition of said animal.

6. A method according to claim 5 wherein said QTL comprises at least a part of an insulin-like growth factor-2 (IGF2) gene.

7. A method according to anyone of claims 1 to 6 wherein in the pig said QTL comprises a marker characterised as nt241(G-A) or as Swc9, as identified in figure 4.

8. A method according to anyone of claims 1-7 wherein a paternal allele of said QTL is predominantly expressed in said animal.

9. A method according to anyone of claims 1-7 wherein a maternal allele of said QTL is predominantly expressed in said animal.

10. An isolated and/or recombinant nucleic acid comprising a parentally imprinted quantitative trait locus (QTL) or functional fragment derived thereof.

11. An isolated and/or recombinant nucleic acid comprising a synthetic parentally imprinted quantitative trait locus (QTL) derived from at least one chromosome or functional fragment derived thereof.

12. A nucleic acid according to claim 10 or 11 at least partly derived from a Sus scrofa chromosome.

13. A nucleic acid according to claim 12 wherein said nucleic acid is at least partly derived from a Sus scrofa chromosome 2, preferably from a region mapping at around position 2p1.7.

14. A nucleic acid according to any one of claims 10 to 13 wherein said QTL is related to the potential muscle mass and/or fat deposition of said animal.

15. A nucleic acid according to any one of claims 10 to 14 wherein said QTL comprises at least a part of a insulin-like growth factor-2 (IGF2) gene.

16. A nucleic acid according to anyone of claims 10 to 15 wherein a paternal allele of said QTL is capable of being predominantly expressed.

17. A nucleic acid according to anyone of claims 10 to 16 wherein a maternal allele of said QTL is capable of being predominantly expressed.

18. Use of a nucleic acid or fragment derived thereof according to claim 10 in a method according to anyone of claims 1-9.

19. Use according to claim 18 to select a breeding animal or animal destined for slaughter for having desired genotypic or potential phenotypic properties.

20. Use according to claim 19 wherein said properties are related to muscle mass and/or fat deposition.

21. An animal such as pig selected by a use according to claim 18 to 20.

22. A animal according to claim 21 characterised in being homozygous for an allele at a paternally imprinted QTL, preferably located at a Sus scrofa chromosome 2 mapping at around position 2p1.7.

23. An animal according to claim 21 or 22 wherein said QTL is related to the potential muscle mass and/or fat deposition of said pig and/or wherein said QTL comprises at least a part of a insulin-like growth factor-2 (IGF2) allele.

24. A transgenic animal comprising a nucleic acid according to anyone of claims 11 to 16.

25. An animal according to anyone of claims 21-24 which is a male.

26. Sperm or an embryo derived from an animal according to anyone of claims 21-25.

27. Use of a sperm or an embryo according to claim 26 in breeding animals destined for slaughter.

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ABSTRACT

The invention relates to methods to select breeding animals or animals destined for slaughter for having desired genotypic or potential phenotypic properties, in particular related to muscle mass and/or fat deposition. The invention provides a method for selecting a pig for having desired genotypic or potential phenotypic properties comprising testing a sample from said pig for the presence of a quantitative trait locus (QTL) located at a Sus scrofa chromosome 2 mapping at position 2p1.7.

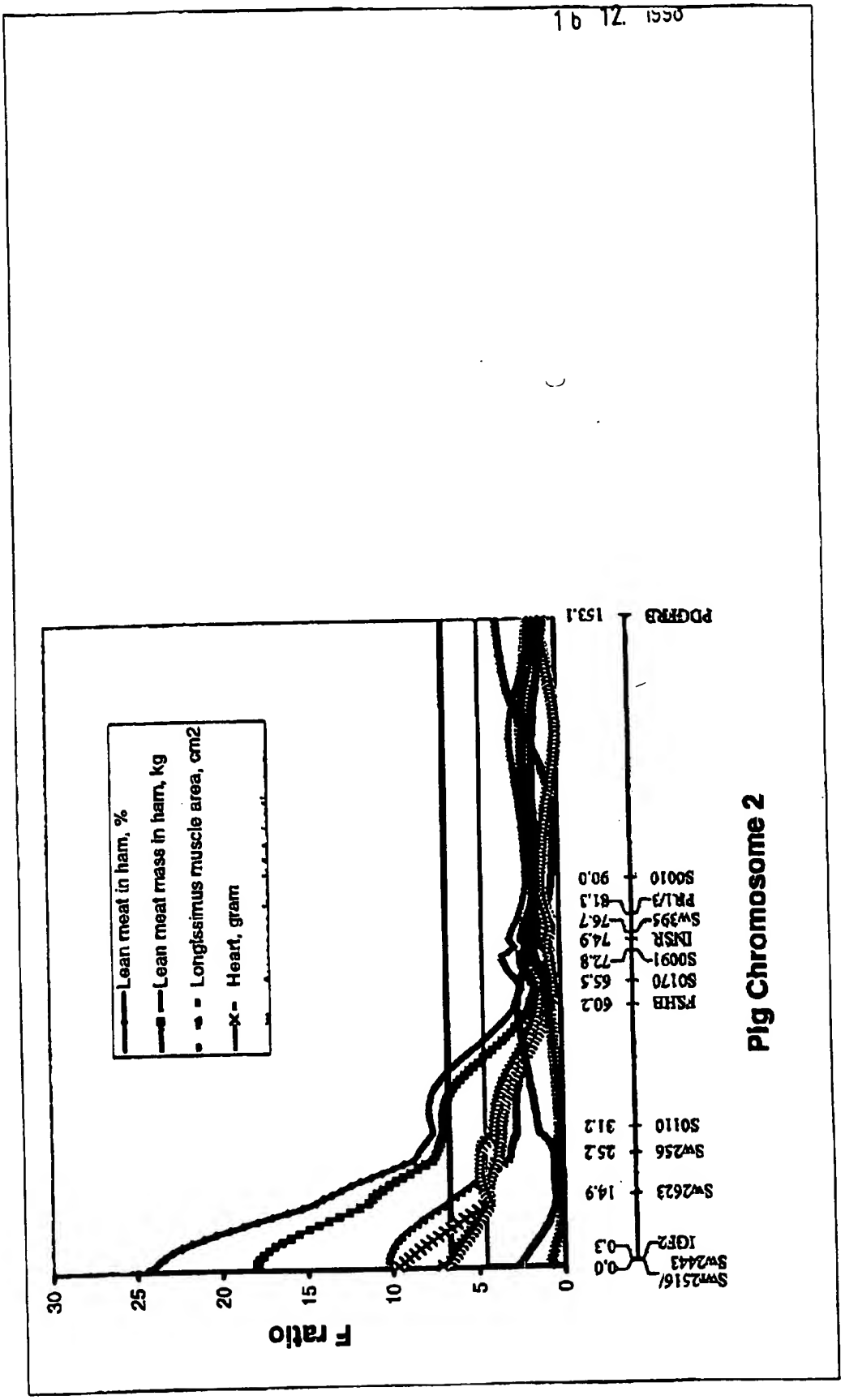

16. 12. 1998
Lean meat in ham, %
Lean meat mass in ham, kg
Longissimus muscle area, cm2
Heart, gram
F ratio
30
25
20
15
10
5
0
Pig Chromosome 2
PDGFRB 153.1
S0010 90.0
PRL/3 81.3
Sw395 76.7
DNSR 74.9
S0091 72.8
S0170 65.5
FSHB 60.2
S0110 31.2
Sw256 25.2
Sw2623 14.9
IGF2 0.3
Sw2443 0.0
Sw2516/

Figure 1

Figure 2

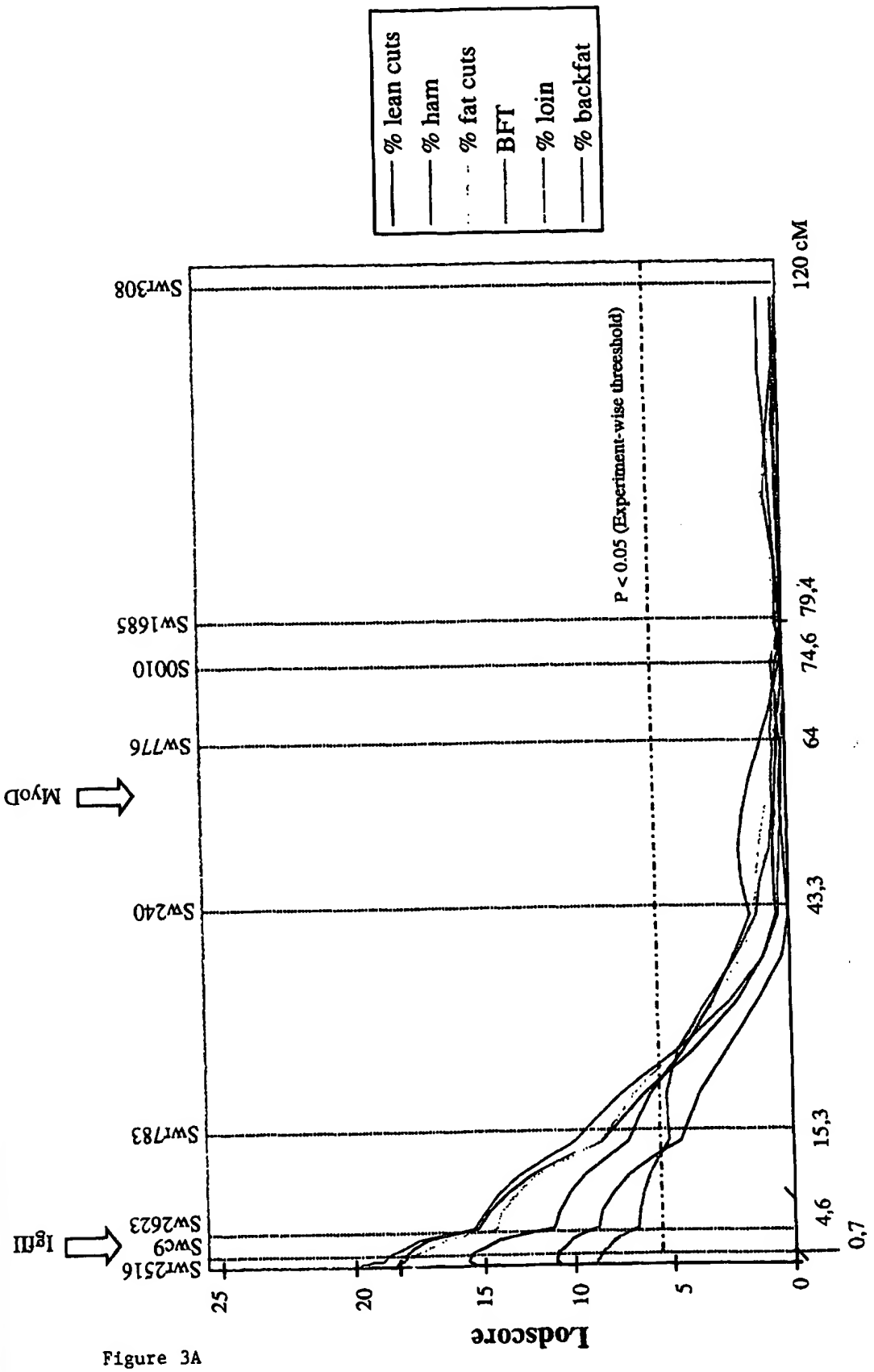

% lean cuts
% ham
% fat cuts
BFT
% loin
% backfat
120 cM
P < 0.05 (Experiment-wise threshold)
Sw308
Sw1685
S0010
Sw776
MyoD
Sw240
Sw783
Ig[II
Sw2623
Swc9
Sw2516
79,4
74,6
64
43,3
15,3
4,6
0,7
25
20
15
10
5
0
Lodscore

Figure 3A

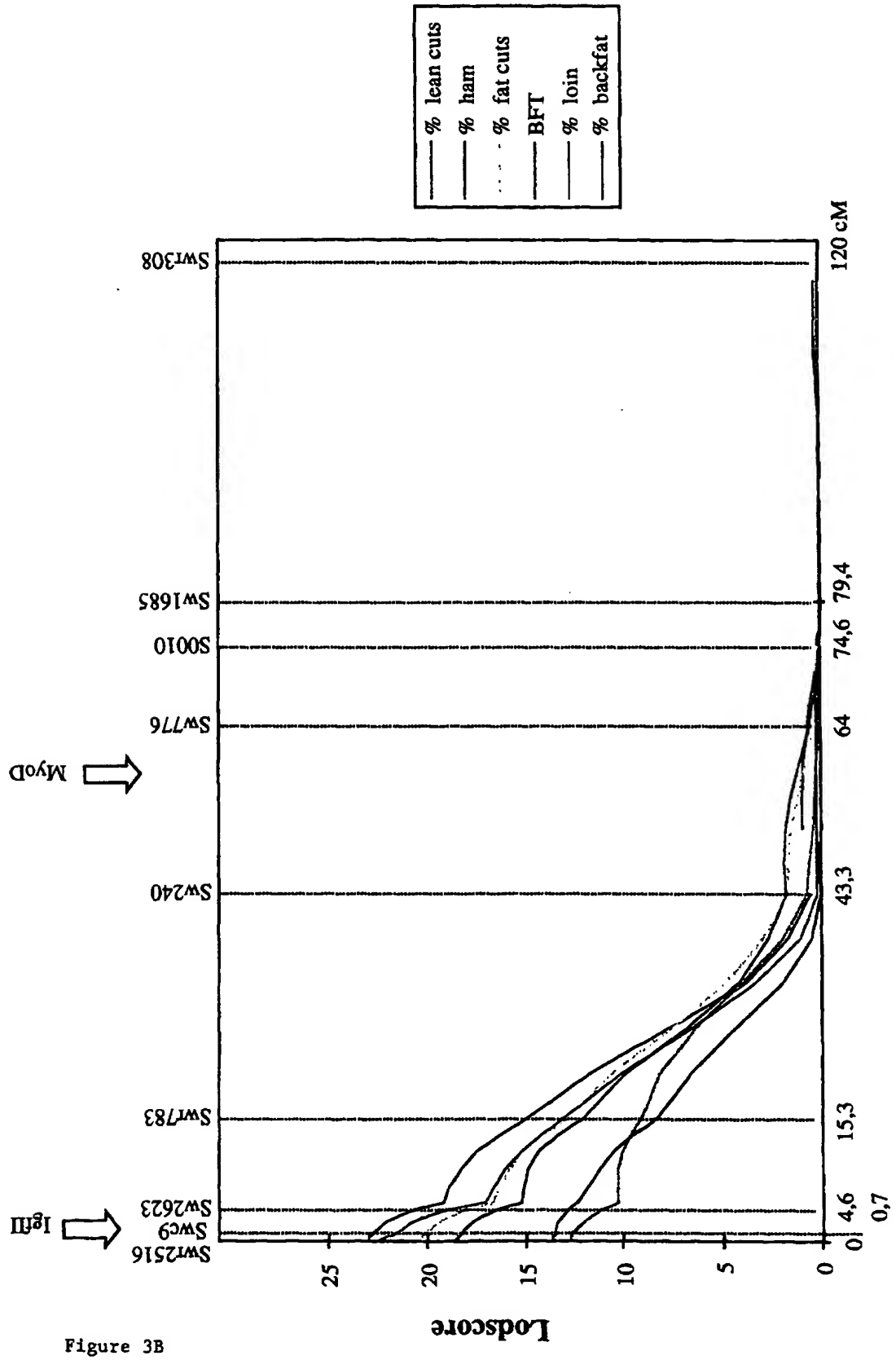

% lean cuts
% ham
% fat cuts
BFT
% loin
% backfat
120 cM
Sw1308
Sw1685
S0010
Sw776
Sw240
Sw783
Sw2623
Sw2516
Sw9
IgfII
MyoD
79,4
74,6
64
43,3
15,3
4,6
0,7
0
5
10
15
20
25
Lodscore

Figure 3B

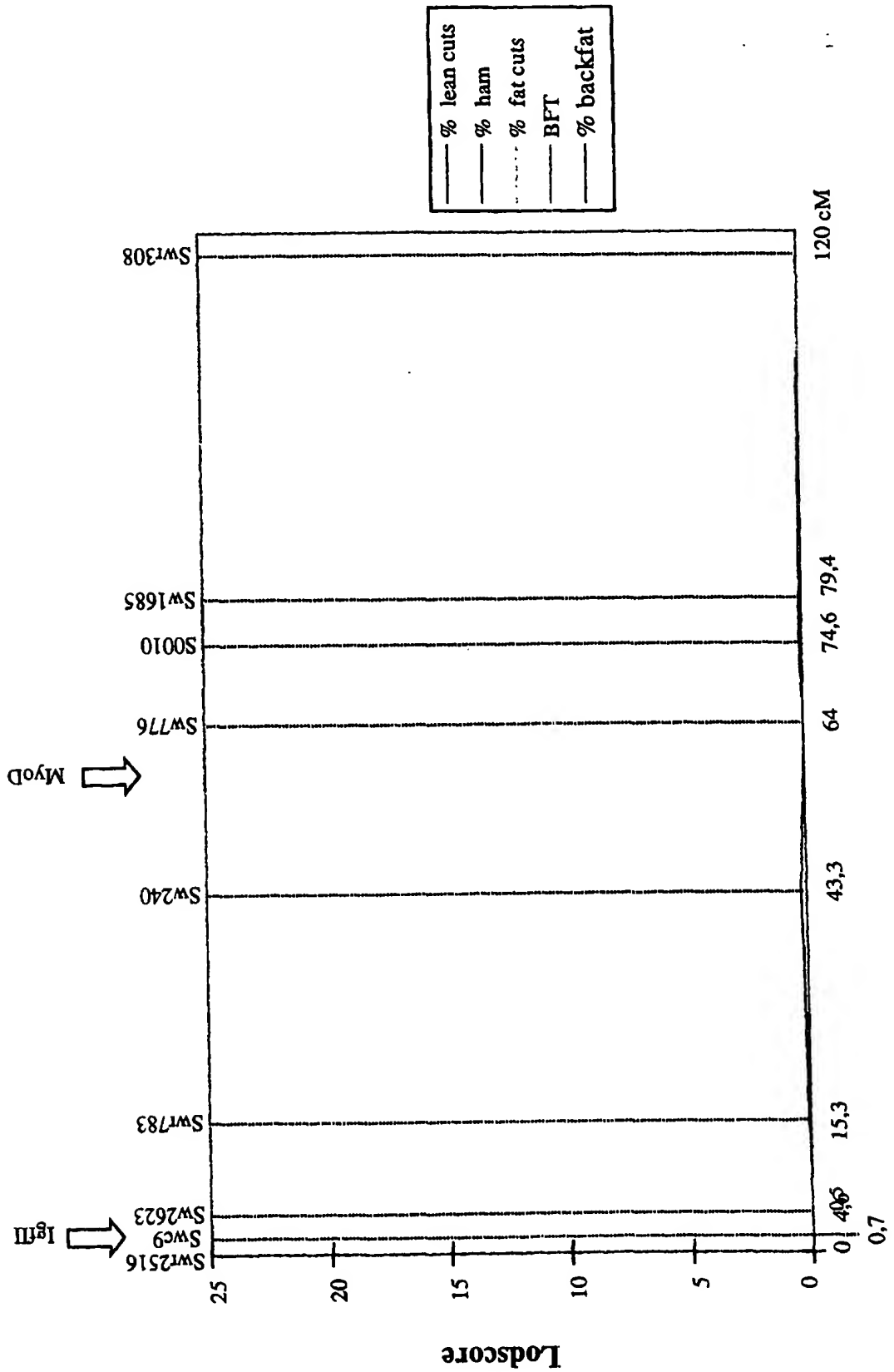
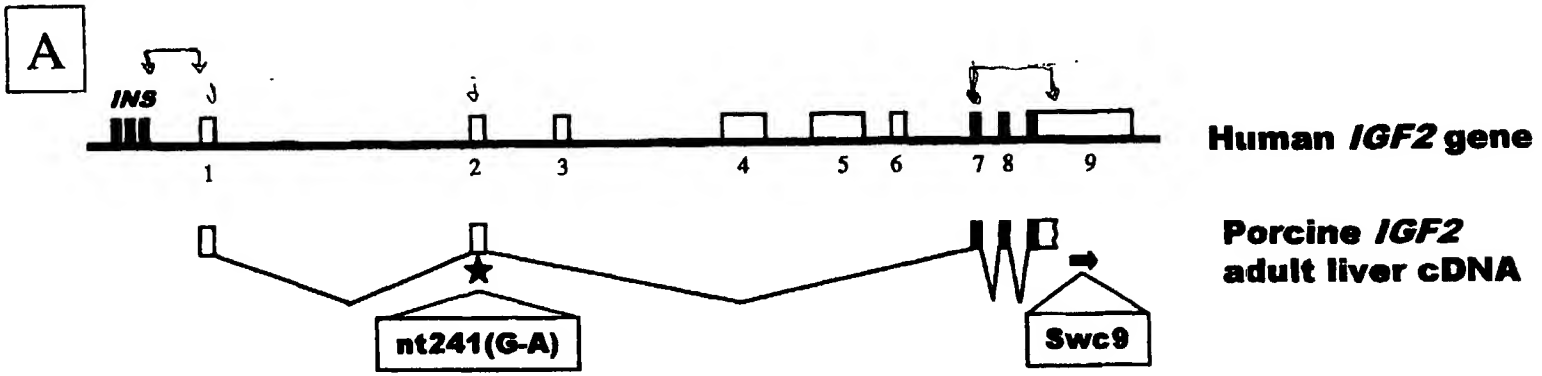

% lean cuts
% ham
% fat cuts
BFT
% backfat
120 cM
79,4
74,6
64
43,3
15,3
4,5
0
0,7
Sw308
Sw1685
S0010
Sw776
Sw240
Sw783
Sw2623
Sw9
Sw2516
MyoD
IgfII
25
20
15
10
5
0
Lodscore

Figure 3C



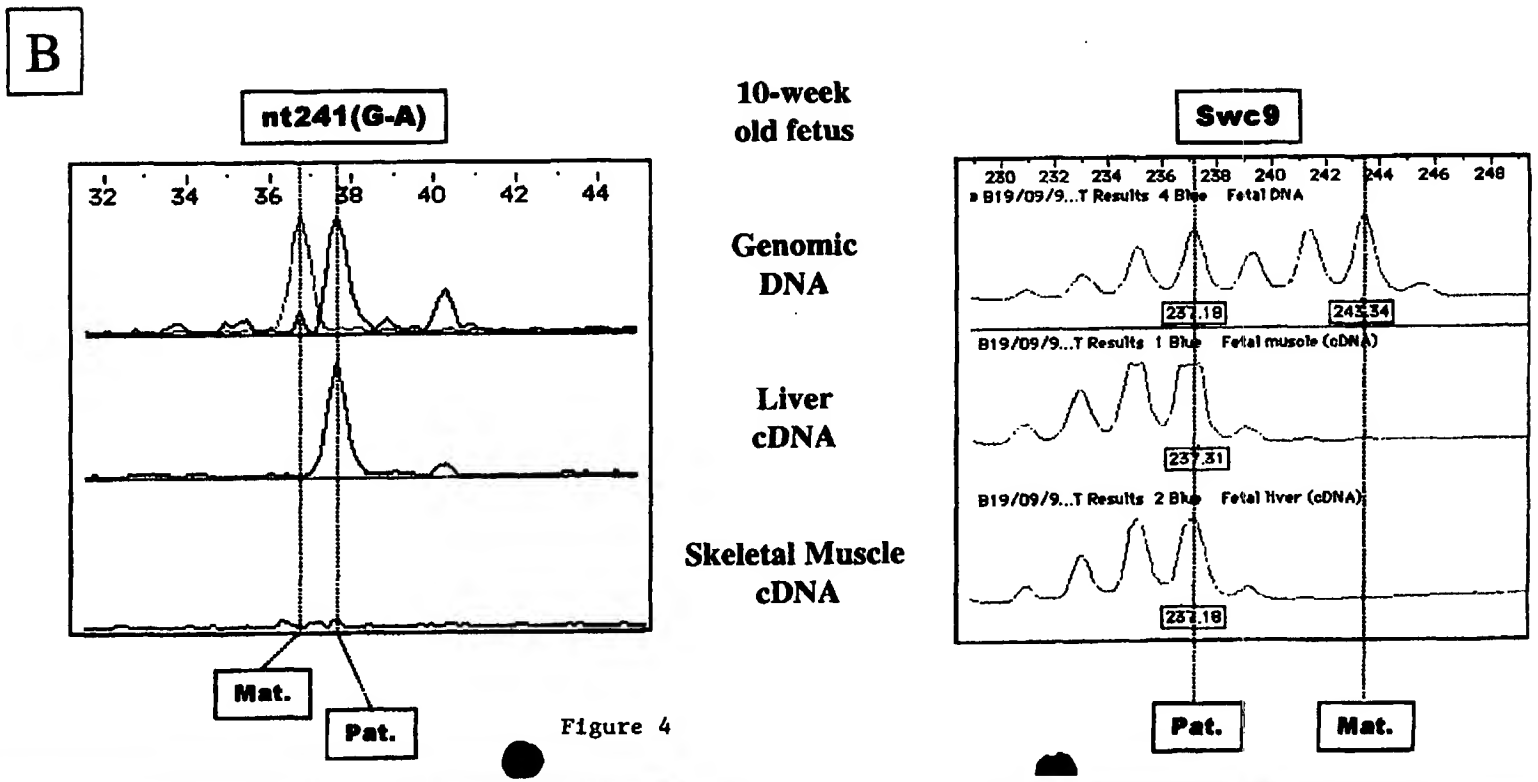


Figure 4